Cell, Vol. 113, 37–47, April 4, 2003, Copyright ©2003 by Cell Press

Migfilin and Mig-2 Link Focal Adhesions to Filamin and the Actin Cytoskeleton and Function in Cell Shape Modulation

Yizeng Tu, Shan Wu, Xiaohua Shi, Ka Chen and Chuanyue Wu*
Department of Pathology
University of Pittsburgh
Pittsburgh, Pennsylvania 15261

Summary

Cell-extracellular matrix adhesion is an important determinant of cell morphology. We show here that migfilin, a LIM-containing protein, localizes to cell-matrix adhesions, associates with actin filaments, and is essential for cell shape modulation. Migfilin interacts with the cell-matrix adhesion protein Mig-2 (mitogen inducible gene-2), a mammalian homolog of UNC-112, and the actin binding protein filamin through its C- and N-terminal domains, respectively. Loss of Mig-2 or migfilin impairs cell shape modulation. Mig-2 recruits migfilin to cell-matrix adhesions, while the interaction with filamin mediates the association of migfilin with actin filaments. Migfilin therefore functions as an important scaffold at cell-matrix adhesions. Together, Mig-2, migfilin and filamin define a connection between cell matrix adhesions and the actin cytoskeleton and participate in the orchestration of actin assembly and cell shape modulation.

Introduction

Cell morphology is controlled to a large extent by the coordinated organization of the extracellular matrix (ECM), plasma membrane, and the cytoskeleton. Protein complexes at the ECM-membrane-actin cytoskeleton junctions (e.g., focal adhesions and fibrillar adhesions) provide physical connections between the extracellular and intracellular compartments and orchestrate actin cytoskeleton organization, cell shape modulation, and other fundamental cellular processes (Hynes, 1992; Burridge and Chrzanowska-Wodnicka, 1996; Calderwood et al., 2000; Geiger et al., 2001). Receptors of integrin family provide a major transmembrane linkage at the cell-ECM adhesions (Hynes, 1992; Tamkun et al., 1986). The connections from integrins to the actin cytoskeleton, however, are complex and their molecular basis is incompletely understood.

Two approaches, one based on protein interactions identified biochemically or by yeast two-hybrid library screens and the other based on classic or reverse genetics, have been proven fruitful in identifying protein complexes that are critical in the coupling of transmembrane integrin receptors to the actin cytoskeleton. Studies based on interactions with the cytoplasmic domains of integrins, actin, or other components of the integrin-actin structures have identified a number of proteins that mediate the connection between integrin and the actin cytoskeleton (Liu et al., 2000; Zamir and Geiger, 2001). Independent genetic studies in model organisms such as *C. elegans* and *Drosophila* have also led to the identification of a subset of mutants that are defective in the linkage between integrins and the actin cytoskeleton (Brown, 2000; Williams and Waterston, 1994). Characterization of these mutants in the invertebrate model organisms has identified several genes that are critically involved in the connection of integrins to the actin cytoskeleton (Hobert et al., 1999; Rogalski et al., 2000; Brown et al., 2000; Zervas et al., 2001; Brown et al., 2002; Mackinnon et al., 2002; Zervas and Brown, 2002). It is particularly exciting that a common set of proteins including talin/rhea (Horwitz et al., 1986; Brown et al., 2002), ILK/PAT-4 (Hannigan et al., 1996; Zervas et al., 2001; Mackinnon et al., 2002), PINCH/UNC-97 (Hobert et al., 1999; Tu et al., 1999), and members of the CH-ILKBP/actopaxin/affixin/parvin/PAT-6 family (Tu et al., 2001; Nikolopoulos and Turner, 2000; Olski et al., 2001; Yamaji et al., 2001; Wu and Dedhar, 2001) have been identified independently using these two different approaches, confirming the importance of these proteins in this process.

Recent genetic studies have identified *unc-112*, which encodes a novel FERM domain-containing protein, as an essential gene for the assembly of integrin-containing muscle attachment structures in *C. elegans* (Rogalski et al., 2000). Mitogen inducible gene-2 (Mig-2) (Wick et al., 1994) is a mammalian protein that shares significant sequence homology with *C. elegans* UNC-112. The functions of Mig-2, however, were not known. We show here that Mig-2 is a component of the ECM adhesion structures in mammalian cells. Furthermore, small interfering RNA (siRNA) mediated gene silencing, a powerful approach of reverse genetics (for recent reviews, see Elbashir et al., 2002; Hannon, 2002; Hudson et al., 2002), demonstrates that Mig-2 is indispensable for proper control of cell shape change. To gain insight into the molecular mechanism by which Mig-2 functions, we carried out yeast two-hybrid screens aimed at identifying proteins that interact with Mig-2. Our results show that Mig-2 interacts with a novel LIM domain-containing protein (termed as migfilin). Migfilin colocalizes with Mig-2 at cell-ECM adhesion sites and associates with actin filaments. Consistent with the binding of migfilin to Mig-2, Mig-2 serves as a docking site recruiting migfilin to cell-ECM adhesion sites. Importantly, loss of migfilin, like that of Mig-2, results in a defect in the cellular control of shape change. Thus, migfilin not only physically interacts with Mig-2 and colocalizes with Mig-2 at cell-ECM adhesions but also is functionally coupled to Mig-2. To gain further insight into the mechanism, we have screened for proteins that interact with migfilin. Our results show that migfilin binds to filamin, an actin cross-linking protein that is crucial for actin cytoskeleton organization (for recent reviews, see Stossel et al., 2001; van der Flier and Sonnenberg, 2001). Migfilin interacts with filamin and Mig-2 via its N-terminal and C-terminal domains, respectively. While Mig-2 mediates the localization of migfilin to cell-ECM adhesion sites, the interaction of migfilin with filamin mediates the migfilin

*Correspondence: carywu@pitt.edu

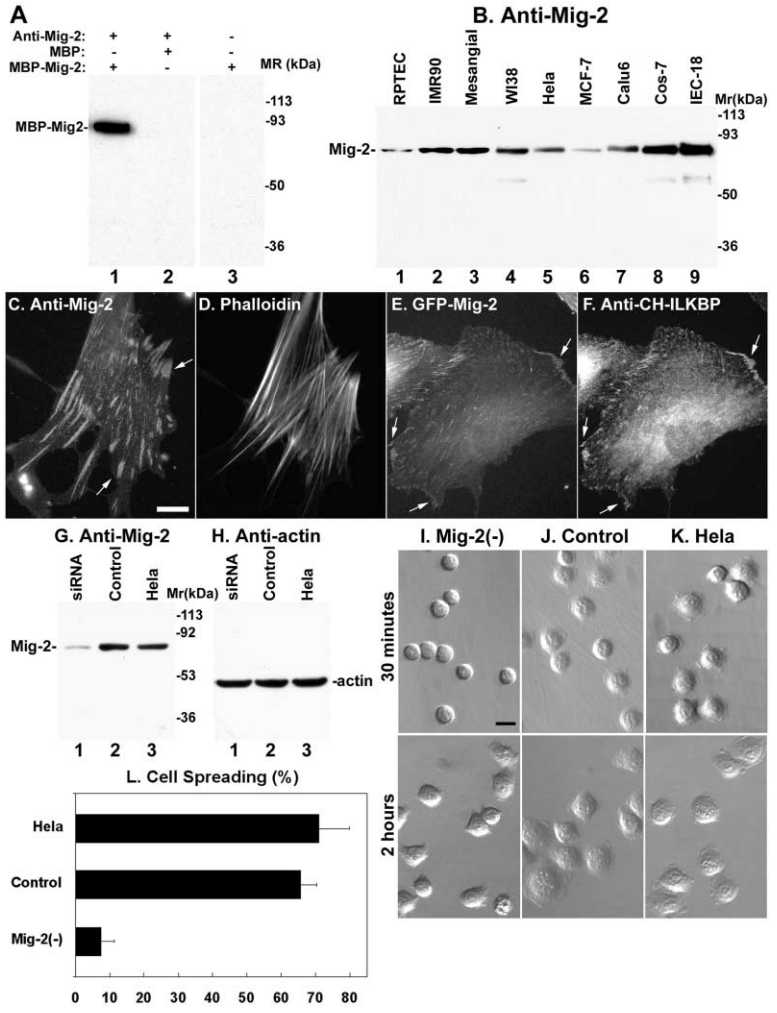

Figure 1. Mig-2 Localizes to Cell-ECM Adhesions and Is Essential for Cell Shape Modulation

(A) Monoclonal anti-Mig-2 antibody. MBP fusion protein containing Mig-2 residues 218–486 (lanes 1 and 3) and MBP (lane 2) (0.1 μg protein/lane) were analyzed by Western blotting with anti-Mig-2 mAb 3A3 (lanes 1 and 2) or an irrelevant mouse IgG as a control (lane 3).

(B) Mig-2 expression. Lysates (15 μg protein/lane) of human cells (as indicated in the figure) were analyzed by Western blotting with anti-Mig-2 mAb 3A3.

(C–F) Subcellular localization. Human WI-38 cells were dually stained with anti-Mig-2 mAb 3A3 (C) and phalloidin (D). In (E and F), mouse C2C12 cells were transfected with GFP-Mig-2 expression vector and stained with mouse mAb 1D4 recognizing focal adhesion protein CH-ILKBP (Tu et al., 2001) and Rhodamine Red^{TX}-conjugated anti-mouse IgG antibodies. Note that endogenous Mig-2 (C) and GFP-Mig-2 (E) are clustered at cell-ECM adhesion sites (arrows). Bar is equal to 10 μm.

(G and H) RNAi suppression of Mig-2 expression. The expression of Mig-2 in the parental Hela cells (lane 3), Mig-2 siRNA (lane 1) or the control RNA (lane 2) transfectants (20 μg protein/lane) were analyzed by Western blotting with anti-Mig-2 mAb 3A3 (G) or an anti-actin antibody (H).

(I–L) Cell spreading. The parental Hela cells (K), Mig-2 siRNA (I) and the control RNA (J) transfectants were allowed to spread on fibronectin-coated plates for 30 min or two hours. Bar is equal to 35 μm. The percentage of cells adopting spread morphology 30 min after plating was quantified by analyzing more than 300 cells from four randomly selected fields (L). Data represent means ± SD. Depletion of Mig-2 results in a similar defect on cell spreading on laminin (not shown in the figure).

association with actin filaments. Depletion of migfilin results in a significant reduction of sedimentable actin. These results reveal a novel scaffolding complex that links the actin filaments to the integrin-containing cell-ECM adhesions and suggest a crucial role of this linkage in the orchestration of actin cytoskeleton organization and cell shape change.

Results

Mig-2 Is a Component of the Cell-ECM Adhesion Structures

We cloned a full-length cDNA encoding Mig-2 from a human lung cDNA library. To facilitate studies on Mig-2, we generated monoclonal antibodies (mAbs) that specifically recognize Mig-2. To do this, we generated GST- and MBP-fusion proteins and used the GST-Mig-2 fusion protein as an antigen to immunize mice. Mouse mAbs were screened by ELISA and Western blotting using the MBP-Mig-2 fusion protein. The mAbs that recognize MBP-Mig-2 (Figure 1A, lane 1) but not MBP (Figure 1A, lane 2) were selected. Western blotting analyses with anti-Mig-2 mAbs showed that Mig-2 is expressed in a variety of human cell types (Figure 1B). To determine the subcellular localization of Mig-2, we immunofluorescently stained human cells with the anti-Mig-2 mAbs. The results showed that Mig-2 was co-aligned with actin stress fibers and clustered at cell-ECM adhesion sites where actin stress fibers were anchored (Figures 1C and 1D). To confirm this, we expressed a GFP-Mig-2 protein in mammalian cells. Staining of the GFP-Mig-2 expressing cells with a mAb recognizing CH-ILKBP, a component of cell-ECM adhesions (Tu et al., 2001), confirmed that GFP-Mig-2 localizes to cell-ECM adhesion sites (Figures 1E and 1F, arrows).

Mig-2 Is Required for Cell Shape Modulation

To assess the function of Mig-2, we employed RNA-mediated interference (RNAi) to suppress the expression of Mig-2 in human cells. We introduced a small (21-nucleotide) Mig-2 interfering RNA and a 21-nucleotide irrelevant RNA as a control, respectively, into HeLa cells. Western blotting analyses showed that transfection with the Mig-2 siRNA (Figure 1G, lane 1), but not that of the control RNA (Figure 1G, lane 2), effectively suppressed the expression of Mig-2. Equal loading of the cell lysates

was confirmed by probing the same samples with an anti-actin antibody (Figure 1H). Majorities of the parental Hela cells and the control transfectants exhibited spread morphology within 30 min of plating (Figures 1J and 1K). By contrast, cells that were deficient of Mig-2 remained round at the same time point (Figures 1I and 1L), albeit they were able to spread after much longer incubation (Figure 1I). These results indicate that Mig-2 is required for proper control of cell spreading.

Identification of Migfilin, a Novel Mig-2 Binding Protein

To gain insight into the molecular mechanism by which Mig-2 functions in cell shape modulation, we sought to identify proteins that interact with Mig-2. Yeast two-hybrid screens of a human lung cDNA library showed that Mig-2 interacts with migfilin, a novel protein that consists of three LIM domains at the C terminus, an N-terminal region, and a proline-rich region located between the C- and N-terminal regions (Figure 2A). PCR analyses of the human lung cDNA library revealed that there exist two different lengths of migfilin cDNAs. Sequencing of the cDNAs showed that the shorter migfilin(s) cDNA encodes a protein that is identical to migfilin except that it lacks the proline-rich region, suggesting that migfilin(s) likely represents a splicing variant of migfilin. Search of the GenBank confirmed that there exist cDNAs in other human tissues corresponding to both forms of migfilin. cDNAs encoding proteins that are homologs to human migfilin are present in other species including mouse (mouse migfilin, GenBank accession number AAH0477, is 78% identical to human migfilin at the amino acid sequence level). Both forms of migfilin interacted with Mig-2 (Figure 2B). Furthermore, studies with migfilin deletion mutants showed that the C-terminal LIM-containing region, but not the N-terminal region, interacted with Mig-2 (Figure 2B). GST-fusion protein pull-down experiments showed that migfilin was readily coprecipitated with GST-Mig-2 fusion protein (Figure 2C, lane 3) but not GST (Figure 2C, lane 2), confirming the interaction between Mig-2 and migfilin.

Migfilin Colocalizes with Mig-2 at Cell-ECM Adhesions and Associates with the Actin Filaments

To facilitate studies on the subcellular localization of migfilin, we generated anti-migfilin mAbs. To do this, we produced GST- and MBP-migfilin fusion proteins, immunized mice with GST-migfilin, and screened the hybridomas with MBP-migfilin. Mouse mAbs recognizing MBP-migfilin but not MBP were selected. To further test the activity of the anti-migfilin mAbs, we expressed FLAG-tagged migfilin (Figure 2D, lane 3) and migfilin(s) (Figure 2D, lane 2), respectively, in Chinese hamster ovary (CHO) cells. Probing the cell lysates with anti-migfilin mAbs confirmed that they recognize both forms of migfilin (Figure 2E, lanes 2 and 3). Interestingly, the mouse anti-migfilin mAbs failed to detect migfilin in hamster (Figure 2E, lane 1), mouse, or rat cells (data not shown). We obtained hybridomas that produce mAbs recognizing both human and murine migfilin during initial screens but none of these hybridomas was stable during subcloning. Migfilin was detected in a variety of human cells including WI-38 (Figure 2F, lane 1), Hela, MCF-7, Calu6, RD, A-431, IMR-90, HT-1080, 293 cells, and primary cells such as kidney glomerular mesangial and proximal tubule epithelial cells (not shown), indicating that migfilin, like Mig-2, is widely expressed in human cells. Intriguingly, migfilin(s) was not detected in many of the cell types that were analyzed, although a very weak band with a predicted size of migfilin(s) was detected in WI-38 cells (Figure 2F, lane 1) and HeLa cells (not shown).

To analyze the subcellular localization of migfilin, we stained WI-38 cells with the anti-migfilin mAbs. Migfilin was associated with actin stress fibers and clustered at cell-ECM adhesions where actin stress fibers were anchored (Figures 2G and 2H). Double staining of cells with rhodamine-conjugated anti-migfilin and FITC-conjugated anti-Mig-2 mAbs confirmed that migfilin was colocalized with Mig-2 in cell-ECM adhesions (Figures 2I and 2J). To analyze the subcellular localization of migfilin variants, we expressed human migfilin and migfilin(s) in mouse C2C12 cells and stained the cells with a mAb that recognizes human but not mouse migfilin. The results showed that both migfilin variants were associated with actin stress fibers and clustered at cell-ECM adhesion sites (Figures 2K–2N). We have observed intracellular aggregates of migfilin(s), which was probably caused by the overexpression of migfilin(s) in the transfectants (Figure 2M). Interestingly, these aggregates were also positively stained by phalloidin (Figure 2N), indicating that they also contain F actin.

Mig-2 Recruits Migfilin to Cell-ECM Adhesions

The findings that migfilin binds to Mig-2 and colocalizes with Mig-2 at cell-ECM adhesions prompted us to test whether Mig-2 functions as a docking site recruiting migfilin to cell-ECM adhesions. We reasoned that if Mig-2 indeed functions as a migfilin-docking site, the Mig-2 binding C-terminal fragment of migfilin, but not the N-terminal fragment of migfilin that does not bind to Mig-2, should localize to Mig-2-rich cell-ECM adhesions. Furthermore, migfilin should not localize to cell-ECM adhesions in the absence of Mig-2. To test this, we expressed GFP-tagged migfilin C- and N-terminal fragments, respectively, in C2C12 cells. The C-terminal fragment of migfilin was clustered at cell-ECM adhesions where abundant Mig-2 was detected (Figures 3A and 3B). By contrast, the N-terminal fragment of migfilin was not coclustered with Mig-2 in cell-ECM adhesions although it associated with the actin filaments (Figures 3C and 3D). These results provide strong evidence for a crucial role of Mig-2 in the recruitment of migfilin to cell-ECM adhesions. To further test this, we suppressed Mig-2 expression by RNAi (Figure 1G) and stained the Mig-2 deficient cells with mAbs specific for migfilin, Mig-2, and paxillin (as a marker of focal adhesions), respectively. As expected, clusters of Mig-2 were detected at cell-ECM adhesions in the control cells (Figure 3E) but not in the Mig-2 deficient cells (Figure 3F). Staining of the cells with an anti-paxillin antibody revealed that paxillin was clustered at focal adhesions in the Mig-2 deficient cells (Figure 3H) as well as in the control cells (Figure 3G), indicating that Mig-2 is not absolutely required for the formation of cell-ECM adhesion struc-

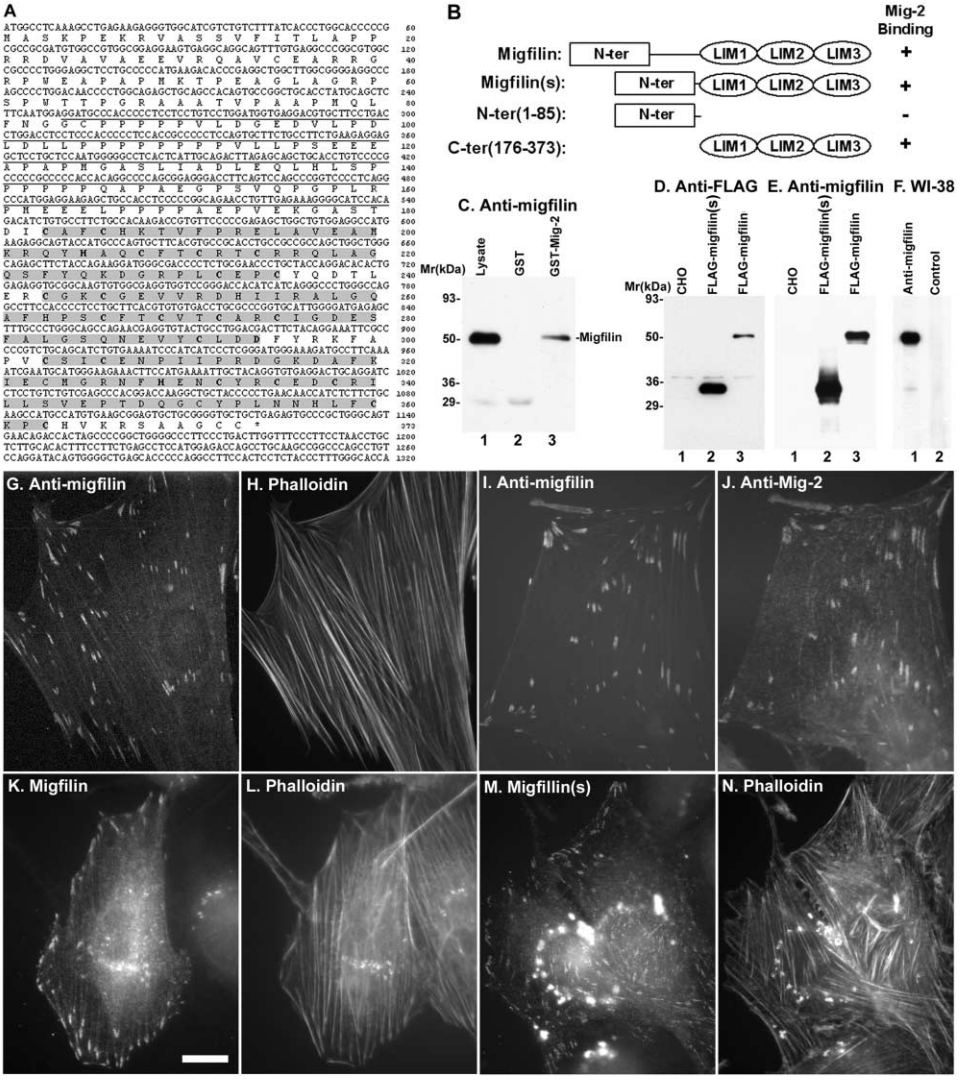

Figure 2. Identification and Subcellular Localization of Migfilin

(A) Nucleotide and amino acid sequences of human migfilin. The amino acid sequence is shown below the nucleotide sequence and the asterisk indicates the stop codon (GenBank accession number AY180161). The LIM domains are indicated by gray background. The nucleotides encoding the proline-rich region (residues 84–180, InterPro accession number IPR000694), which is absent in migfilin(s), are underlined.

(B) The Mig-2-migfilin interaction. The interaction was determined by yeast two-hybrid binding assays as described in the Experimental Procedures. The numbers in parentheses indicate migfilin residues.

(C) GST-fusion protein pull-downs assay. WI-38 cell lysates (230 μg) were incubated with equal amount (10 μg) of GST (lane 2) or GST-Mig-2 fusion protein (lane 3). GST and GST-Mig-2 were precipitated with glutathione-Sepharose 4B beads. Migfilin was detected by Western blotting with an anti-migfilin mAb (clone 43). Lane 1, WI-38 cell lysates (8.6 μg/lane).

(D and E) Lysates (15 μg/lane) of CHO cells that were transfected with a FLAG control vector lacking migfilin sequence (lane 1) or vectors encoding FLAG-tagged migfilin(s) (lane 2) or migfilin (lane 3) were analyzed by Western blotting with anti-FLAG (D) or anti-migfilin (E) mAbs.

(F) WI-38 cell lysates (10 μg/lane) were probed with the anti-migfilin mAb (lane 1) or a control mouse IgG (lane 2).

(G–N) Subcellular localization. WI-38 cells were dually stained with the anti-migfilin mAb (G) and phalloidin (H), or dually stained with rhodamine-conjugated anti-migfilin (I) and FITC-conjugated anti-Mig-2 (J) mAbs.

(K–N) Mouse C2C12 cells were transfected with vectors encoding FLAG-migfilin (K and L) or FLAG-migfilin(s) (M and N). The cells were dually stained with the anti-migfilin mAb (K and M) and phalloidin (L and N). Bar is equal to 10 μm.

tures. Strikingly, unlike in the control cells in which migfilin is clustered at focal adhesions (Figure 3I), migfilin was not clustered at focal adhesions but was instead present rather diffusely in the Mig-2 deficient cells (Figure 3J). This was confirmed by double staining of the Mig-2 deficient cells with anti-paxillin (Figure 3K) and anti-migfilin antibodies (Figure 3L). Thus, while Mig-2 is not absolutely required for the formation of focal adhesions, it is required for the recruitment of migfilin into focal adhesions.

Migfilin Is Required for Cell Shape Modulation

We next tested whether migfilin is functionally coupled to Mig-2, which, as shown earlier (Figure 1), is required for proper control of cell shape change. To do this, we suppressed the expression of migfilin by RNAi. Trans-

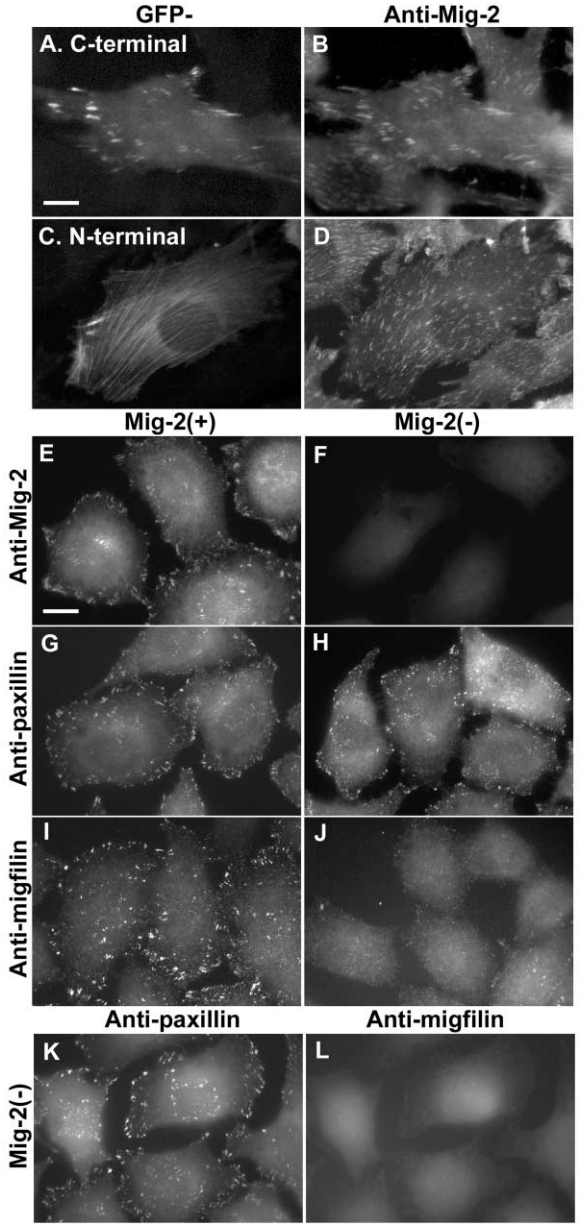

Figure 3. Mig-2 Recruits Migfilin to Cell-ECM Adhesion Sites

(A–D) Mouse C2C12 cells that were transfected with vectors encoding GFP-tagged C-terminal domain (residues 176–373) (A and B) or N-terminal domain (residues 1–85) (C and D) of migfilin were stained with anti-Mig-2 mAb 3A3 and Rhodamine Red^{TX}-conjugated anti-mouse IgG antibodies. GFP fusion proteins and Mig-2 were visualized under a fluorescence microscopy equipped with GFP (A and C) and rhodamine (B and D) filters. Bar is equal to 15 μm.

(E–L) Hela cells transfected with the Mig-2 siRNA (F, H, and J) or the control RNA (E, G, and I) were plated on fibronectin-coated plates for 24 hr and stained with anti-Mig-2 (E and F), paxillin (G and H), and migfilin (I and J) mAbs and FITC-conjugated anti-mouse IgG antibodies. In (K and L), Mig-2 deficient cells were dually stained with FITC-conjugated anti-paxillin (K) and rhodamine-conjugated anti-migfilin (L) mAbs. Bar is equal to 20 μm.

fection of cells with a migfilin-specific siRNA (Figure 4A, lane 2), but not the Mig-2-specific siRNA (Figure 4A, lane 3) or the control RNA (Figure 4A, lane 1), resulted in a near-total loss of migfilin. The expression of other proteins such as paxillin (Figure 4B) or actin (Figure 4D) was not altered by the depletion of either migfilin or Mig-2. Furthermore, depletion of migfilin did not alter the expression of Mig-2 (Figure 4C, lane 2). Thus, migfilin RNAi effectively inhibited the expression of migfilin without altering that of Mig-2. Depletion of migfilin, like that of Mig-2 (Figures 1I–1L), significantly impaired cell spreading (Figures 4E–4G). The migfilin-deficient cells, like Mig-2 deficient cells (Figures 1I–1L and Figures 3E–3L), were able to spread after prolonged incubation (Figures 4E–4J). We have detected clusters of paxillin in the migfilin-deficient cells (Figure 4J), indicating that cell-ECM adhesion structures were formed in these cells. Staining of the migfilin-deficient cells with anti-Mig-2 mAb showed that Mig-2 localized to focal adhesions, albeit it appeared to be somewhat less well-organized comparing to that in the control cells (comparing Figure 4I with Figure 3E), indicating that migfilin is not absolutely required for the localization of Mig-2 to focal adhesions albeit it may influence Mig-2 organization at the adhesion sites. Taken together, these results show that, despite the presence of Mig-2, loss of migfilin results in a defect in cell spreading that is identical to that resulting from the loss of Mig-2, suggesting that migfilin likely works in concert with Mig-2 in cell shape modulation.

Identification of Filamin as a Migfilin Interactive Protein

To gain further insight into the mechanism by which migfilin functions in cell shape modulation, we sought to identify proteins that interact with the N-terminal domain of migfilin, which, as shown earlier (Figure 3C), mediates the association with actin filaments. To do this, we screened a human lung yeast two-hybrid library with the migfilin N-terminal domain as bait. The results showed that the migfilin N-terminal domain interacted with the C-terminal region of filamin A/C encompassing filamin repeats 21 to 24 (Figure 5A). To confirm the interaction, we immunoprecipitated migfilin from human cell lysates with an anti-migfilin mAb (Figure 5B, lane 3). Western blotting analyses of the migfilin immunoprecipitates showed that filamin (Figure 5C, lane 3) was coimmunoprecipitated with migfilin. No filamin (Figure 5C, lane 4) was detected in the control immunoprecipitates lacking migfilin (Figure 5B, lane 4). Thus, consistent with the interaction in yeast, migfilin forms a complex with filamin in mammalian cells.

To map the migfilin binding site on filamin, we generated a series of filamin mutants that contain different filamin repeats. Yeast two-hybrid binding assays showed that deletion of the C-terminal most filamin repeats 23 and 24 did not eliminate the migfilin binding (Figure 5A), indicating that they are not required for the interaction. Analyses of individual filamin repeats showed that repeat 21, but not repeat 20 or 22, interacted with migfilin (Figure 5A). To confirm this, we generated GST-fusion proteins containing different filamin repeats and tested their migfilin binding activity in GST-fusion protein pull-down assays. The results showed that, as expected, GST-fusion protein containing filamin repeats 21–24 (Figure 5D, lane 2), but not GST (Figure 5D, lane 1), bound to migfilin. Furthermore, GST-fusion protein

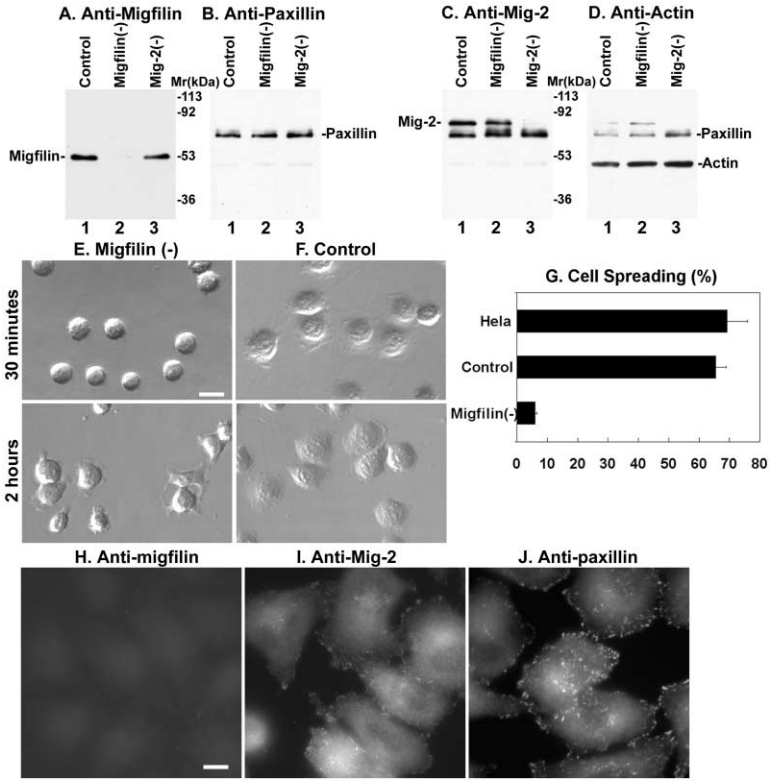

Figure 4. Loss of Migfilin Impairs Cell Shape Change

(A–D) RNAi suppression of migfilin expression. The expression of migfilin in Hela cells that were transfected with the control RNA (lane 1), migfilin siRNA (lane 2) or Mig-2 siRNA (lane 3) (20 μg protein/lane) were analyzed by Western blotting with anti-migfilin (A) or paxillin (B) mAbs. The membrane used in (B) was subsequently reprobed with anti-Mig-2 (C) and actin antibodies (D).

(E–G) Cell spreading. The migfilin siRNA (E) and the control RNA (F) transfectants were allowed to spread on fibronectin-coated plates for 30 min or two hours. Bar is equal to 35 μm. The percentage of the migfilin deficient cells, the control transfectants and the parental Hela cells that adopted spread morphology 30 min after plating was quantified by analyzing more than 300 cells from four randomly selected fields (G). Data represent means ± SD. Depletion of migfilin results in a similar defect on cell spreading on laminin (not shown in the figure).

(H–J) The migfilin deficient cells were plated on fibronectin-coated plates for 24 hr and stained with anti-migfilin (H), Mig-2 (I), or paxillin (J) mAbs. Bar is equal to 20 μm.

containing filamin repeat 21 (Figure 5D, lane 4), but not those containing repeat 20 or 22 (Figure 5D, lanes 3 and 5), bound to migfilin. Thus, consistent with the mutational studies in yeast, filamin repeat 21 mediates the interaction with migfilin.

The Interaction with Filamin Mediates Migfilin Association with the Actin Filaments

What is the function of the migfilin interaction with filamin? The finding that the filamin binding N-terminal domain of migfilin mediates the association with actin filaments (Figure 3C) and the fact that filamin is a component of actin filaments suggest that the interaction with filamin likely mediates the association of migfilin with actin filaments. To test this, we introduced mutations into the N-terminal domain of migfilin and expressed GFP-tagged migfilin mutants in mammalian cells (Figures 6A and 6C). As expected, GFP-fusion proteins containing migfilin (Figures 6A and 6B, lane 5), migfilin(s) (Figures 6A and 6B, lane 6) or the N-terminal fragment of migfilin (Figures 6C and 6D, lane 1), but not GFP (Figures 6A and 6B, lane 4), interacted with filamin. To pinpoint the residues that are critical for the filamin binding, we introduced smaller deletion and point mutations into the N-terminal domain of migfilin. Deletion of the N-terminal most 24 residues (Figures 6C and 6D, lane 4) or substitution of residues K_7R_8 within the N-terminal domain of migfilin with TG (Figures 6C and 6D, lane 3) eliminated the interaction with filamin. Analyses of the cells expressing the GFP-fusion proteins showed that GFP-migfilin and GFP-migfilin(s) associated with actin filaments as well as cell-ECM adhesions (Figures 6E–6H). By marked contrast, the filamin binding defective K_7R_8→TG (Figures 6I and 6J) and ΔN24 mutants (not shown) localized to cell-ECM adhesions but failed to decorate actin filaments that connect the cell-ECM adhesions, indicating that the association of migfilin with actin filaments is indeed mediated by the interaction with filamin.

Migfilin Regulates Actin Assembly In Vivo

It has been well-established that filamin plays a crucial role in the assembly of actin cytoskeleton (reviews in (Stossel et al., 2001; van der Flier and Sonnenberg, 2001)). The findings that migfilin interacts with filamin and associates with actin filaments prompted us to analyze whether migfilin functions in the cellular regulation of actin assembly. To do this, we stained the migfilin deficient cells as well as the control cells that express a normal level of migfilin with phalloidin. Although actin filaments were observed in both the migfilin deficient cells and the control cells, there appeared to be less filamentous actin in the migfilin deficient cells (Figures 7A and 7B). To confirm this, we quantified the ratio of filamentous actin (F actin) versus free globular actin (G actin) in the migfilin deficient cells and the control cells. The results showed that depletion of migfilin significantly reduced the amount of F actin in cells (Figures 7C and 7D), suggesting an important role of migfilin in the cellular regulation of actin assembly.

Discussion

We have identified migfilin as an important molecular scaffold at cell-ECM adhesions participating in the orchestration of actin assembly and cell shape modula-

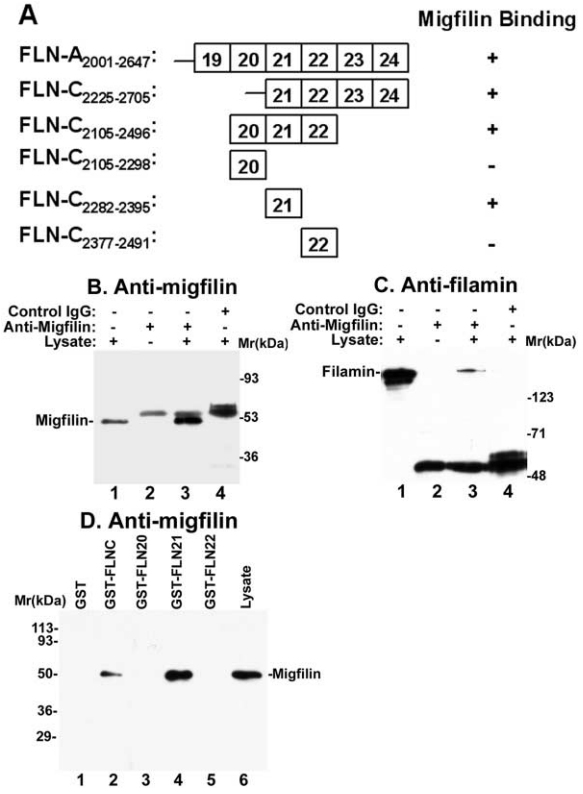

Figure 5. Migfilin Interacts with Filamin

(A) The interaction between migfilin and filamin C-terminal fragments containing different repeats were determined by yeast two-hybrid binding assays as described in the Experimental Procedures.

(B and C) Coimmunoprecipitation of filamin with migfilin. Lysates of human IMR-90 cells were mixed with mouse anti-migfilin mAb or an irrelevant mouse IgG as a control. The migfilin immunoprecipitates (lane 3) or the control precipitates (lane 4) were analyzed by Western blotting with anti-migfilin (B) and filamin (C) mAbs, respectively. Lane 1, IMR-90 cell lysates (9 μg protein/lane). The sample loaded in lane 2 was prepared as that of lane 3 except that the cell lysates were omitted. The bands that migrated slightly slower than the 53 kDa marker in lanes 2–4 were IgG heavy chains derived from the mouse anti-migfilin mAb (lanes 2 and 3) and the control mouse IgG (lane 4).

(D) GST-fusion protein pull-downs. Human 293 cell lysates (280 μg) were incubated with equal amount (10 μg) of GST (lane 1) or GST fusion proteins containing filamin C repeats 21–24 (residues 2225–2705) (lane 2), repeat 20 (residues 2105–2298) (lane 3), repeat 21 (residues 2282–2395) (lane 4), or repeat 22 (residues 2377–2492). GST and GST-filamin fusion proteins were precipitated with glutathione-Sepharose 4B beads. Migfilin was detected by Western blotting with the anti-migfilin mAb. Lane 6, 293 cell lysates (13 μg protein/lane).

tion. Migfilin is shown to cluster at cell-ECM adhesions and associate with actin filaments. At the molecular level, migfilin interacts with Mig-2, a component of cell-ECM adhesions, and filamin, a component of the actin filaments, through its C- and N-terminal domains, respectively. The Mig-2 binding C-terminal domain is necessary and sufficient for mediating the localization of migfilin to cell-ECM adhesions, whereas the filamin binding N-terminal domain is necessary and sufficient for mediating the association of migfilin with actin filaments. These results suggest a model in which migfilin, through its interaction with Mig-2, is recruited to cell-ECM adhesions where it functions as an anchoring site for filamin-containing actin filaments. The functional importance of this anchoring site is manifested by the defect in cell shape modulation caused by the depletion of migfilin. In further support of this model, depletion of Mig-2 prevents the clustering of migfilin in cell-ECM adhesions, resulting in a defect in cell shape modulation that is identical to that induced by the loss of migfilin.

It has been well-established that filamin is essential for cell shape modulation and processes that require extensive shape modulation such as cell migration (Stossel et al., 2001; van der Flier and Sonnenberg, 2001). Filamin A-deficient cells are defective in cell migration (Cunningham et al., 1992). Mutations in the X-linked *FLNa* gene are the causes of human periventricular heterotopia (Fox et al., 1998), an X-linked dominant disorder in which neurons fail to migrate into the cerebral cortex. Consistent with an essential role of filamin in cell migration and morphogenesis, most hemizygous affected males die early during embryogenesis (Fox et al., 1998). Filamin contains an actin binding domain at the N terminus and a number of (24 in human) repeats at the C terminus (Gorlin et al., 1990; Stossel et al., 2001; van der Flier and Sonnenberg, 2001). Filamin mediates at least three functions that are crucial for cell shape modulation and motility: cross-linking of actin filaments, anchoring of actin filaments to cell-ECM adhesion sites, and scaffolding of signaling proteins. Filamin-mediated cross-linking of actin filaments cooperates with other actin binding proteins including Arp2/3 complex promoting actin polymerization (Flanagan et al., 2001; Nakamura et al., 2002; Stossel et al., 2001). Our findings that migfilin associates with actin filaments and that loss of migfilin reduces the level of F actin suggest that migfilin not only provides an important anchoring site for actin filaments at cell-ECM adhesions but also participates in the regulation of filamin-mediated cross-linking and stabilization of actin filaments. Finally, we have mapped the migfilin binding site to the 21 repeat of filamin, which is near (but distinct from) the binding sites of β integrins (Calderwood et al., 2001; Loo et al., 1998; Pfaff et al., 1998; Sharma et al., 1995; van der Flier et al., 2002) and other signaling proteins including RalA, Rho, Rac, Cdc42 (Ohta et al., 1999), Trio (Bellanger et al., 2000), and PAK (Vadlamudi et al., 2002) that are involved in the regulation of the assembly of the integrin-actin structures. Thus, the binding of migfilin to filamin could also play a role in the regulation of the assembly of filamin-containing signaling complexes that control actin assembly, cell morphology, and other fundamental cellular processes.

While it is clear that Mig-2 recruits migfilin to cell-ECM adhesions, it is less clear how Mig-2 is recruited to the adhesion sites. Clusters of Mig-2 were found in focal adhesions in migfilin-deficient cells, suggesting that migfilin is not absolutely required for the focal adhesion localization of Mig-2. Mackinnon et al. recently found that PAT-4/ILK interacts with UNC-112 and is required for the incorporation of UNC-112 into integrin-containing adhesion structures in *C. elegans* (Mackinnon et al., 2002). Consistent with the studies in *C. elegans*, we have found that Mig-2 is able to interact with ILK (Y.T. and C.W., unpublished data). These results raise an interesting possibility that Mig-2 is physically

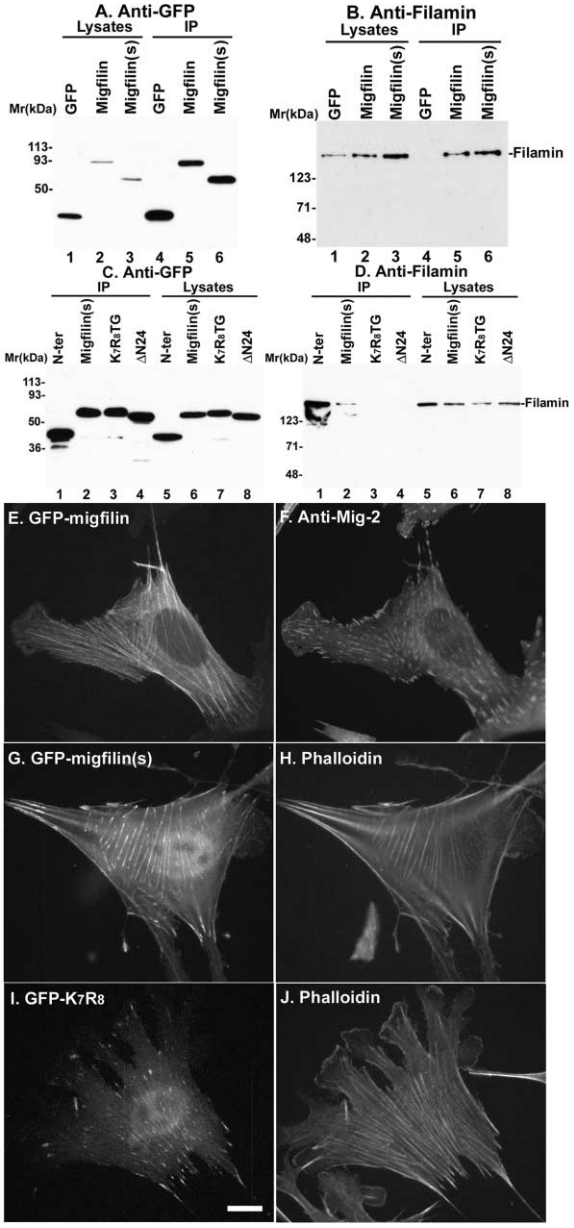

Figure 6. The Interaction with Filamin Mediates the Association of Migfilin with Actin Filaments

(A–D) Filamin binding. In (A and B), lysates of C2C12 cells transfected with vectors encoding GFP (lane 1), GFP-migfilin (lane 2) or GFP-migfilin(s) (lane 3) were mixed with anti-GFP antibodies. The GFP (lane 4), GFP-migfilin (lane 5), and GFP-migfilin(s) (lane 6) immunoprecipitates were analyzed by Western blotting with HRP-conjugated anti-GFP antibodies (A) or mouse anti-filamin mAb and HRP-conjugated anti-mouse IgG antibodies (B). In (C and D), lysates of C2C12 cells transfected with vectors encoding GFP-tagged migfilin(s), ΔN24 mutant in which the N-terminal most 24 residues were deleted, the substitution mutant in which K7R8 were substituted with TG or the migfilin N-terminal domain (residues 1–85) were mixed with anti-GFP antibodies. The cell lysates and anti-GFP immunoprecipitates (as indicated in the figure) were analyzed by Western blotting with HRP-conjugated anti-GFP antibodies (C) or mouse anti-filamin mAb and HRP-conjugated anti-mouse IgG antibodies (D).

(E–J) Association with cell-ECM adhesions and actin filaments. C2C12 cells transfected with vectors encoding the GFP-tagged wild-type and mutant forms of migfilin were stained with rhodamine-phalloidin and mouse anti-Mig-2, respectively. The mouse primary antibody was detected with a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody. (E–J) show representative images of subcellular localization of GFP-tagged migfilin (E and F), migfilin(s) (G and H) and the K7R8 →TG substitution mutant (I and J). Note that GFP-tagged wild-type migfilin proteins, but not the filamin binding defective K7R8 →TG substitution mutant, decorate actin filaments. The filamin binding defective ΔN24 mutant also failed to associate with actin filaments (not shown in the figure), whereas the migfilin N-terminal domain associated with actin filaments (Figure 3C). Bar is equal to 10 μm.

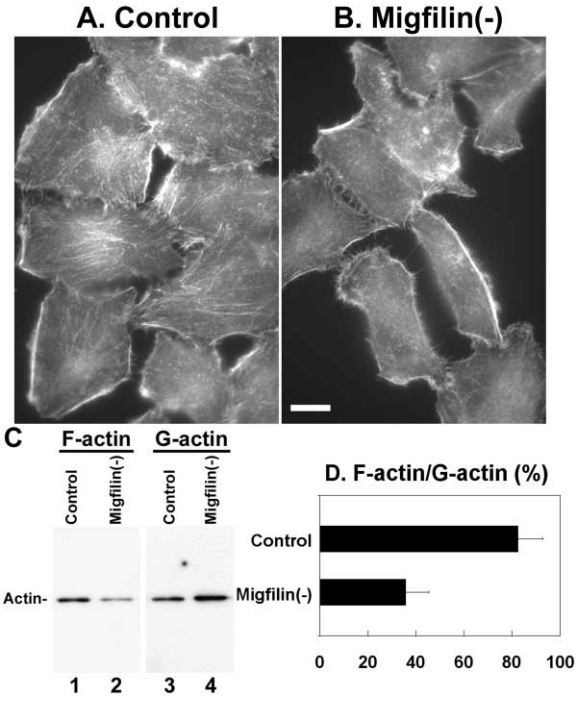

Figure 7. Loss of Migfilin Reduces Actin Assembly In Vivo

(A and B) Hela cells transfected with the control RNA (A) or the migfilin siRNA (B) were stained with phalloidin. Bar is equal to 20 μm.

(C and D) In vivo actin assembly. F actin (lanes 1 and 2) and G actin (lanes 3 and 4) in Hela cells transfected with the control RNA (lanes 1 and 3) or the migfilin siRNA (lanes 2 and 4) were analyzed by Western blotting with anti-actin antibodies (C) and the ratio of F actin versus G actin (D) was quantified as described in the Experimental Procedures. Bars in (D) represent means ± SD from two independent experiments.

and functionally coupled to ILK and consequently integrins in mammalian cells. Clearly, future studies are required to further test this possibility.

Cell-ECM adhesions are complex structures consisting of multiple protein complexes that transduce bidirectional mechanical and chemical signals between extracellular and intracellular compartments (Hynes, 1992; Jockusch et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Calderwood et al., 2000; Critchley, 2000; Geiger et al., 2001; Liu et al., 2000; Turner and Burridge, 1991; Zamir and Geiger, 2001). The interactions mediated by cell-ECM adhesion protein complexes, which couple transmembrane receptors such as integrins to the actin cytoskeleton, are neither linear nor redundant. The presence of multiple cytoplasmic

connections between the integrins and the actin cytoskeletons is necessary for precise regulation of the physical strength of mechanical signals and proper transmission of chemical signals that are essential for cellular control of actin cytoskeleton, shape and other processes (Geiger et al., 2001). The studies presented here suggest that migfilin, through its interactions with Mig-2 and filamin, provides one of such essential cytoplasmic connections at cell-ECM adhesions that regulate actin remodeling and cell morphology.

Experimental Procedures

Cloning of Mig-2

A full-length Mig-2 cDNA was cloned from a human WI-38 lung cDNA library (Clontech) by PCR using the following primers: 5′-CTG GAATTCATGGCTCTGGACGGGATAAGGATGC-3′ and 5′-CAGGTC GACTACAGTTTGGCTAGCTTAAATCAG-3′. The sequence of the Mig-2 cDNA was confirmed by automated DNA sequencing.

Yeast Two-Hybrid Assays

To identify proteins interacting with Mig-2, a cDNA fragment encoding Mig-2 residues 90–680 was inserted into the EcoRI/XhoI sites of a pLexA vector (Clontech). The resulting construct was used as bait to screen a human lung MATCHMAKER LexA cDNA library as we previously described (Tu et al., 2001, 1999, 1998). Four positive plasmids containing cDNA inserts with an identical size (1.3 kb) were selected and sequenced. They contain an identical cDNA fragment encoding the C-terminal fragment of migfilin (residues 206–373). Full-length cDNAs encoding migfilin and migfilin(s) were isolated from the human lung cDNA library by PCR using the following primers: 5′-GTCGAATTCATGGCCTCAAAGCCTGAGAAG-3′ and 5′-CTA CTCGAGTCAGCAGCACCCCGCAGCAC-3′.

To identify proteins interacting with the N-terminal domain of migfilin, a cDNA fragment encoding the migfilin residues 1–92 was inserted into the EcoRI/XhoI sites of the pLexA vector. The resulting construct was used as bait to screen the human lung MATCHMAKER LexA cDNA library. Four positive plasmids, two containing a cDNA insert of 2.3 kb and the other two containing a cDNA insert of 2.5 kb, were selected and sequenced. The results showed that they contain coding sequences of the C-terminal regions of filamin A (residues 2001–2647) and filamin C (residues 2225–2705), respectively.

Additionally, we performed yeast two-hybrid binding assays using purified pLexA and pB42 constructs encoding Mig-2, migfilin and filamin sequences (as specified in each experiment) based on a previously described method (Tu et al., 2001, 1999).

Generation of GST- and MBP-Tagged Mig-2, Migfilin and Filamin Fusion Proteins

DNA fragments encoding Mig-2, migfilin or filamin sequences as specified in each experiment were prepared by PCR and inserted into the pGEX-5x-1 vector (Pharmacia) or the pMAL-C2 vector (New England BioLabs). The recombinant vectors were used to transform *E. coli* cells. The expression of the GST- and MBP-fusion proteins was induced with IPTG, and they were purified by affinity chromatography using glutathione-Sepharose 4B and amylose-agarose, respectively, as we previously described (Tu et al., 2001, 1999).

GST-Fusion Protein Pull-Down Assays

Cells were lysed with 1% TX-100 in 50 mM HEPES [pH 7.1] containing 150 mM NaCl, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10 mM EDTA, and protease inhibitors. The lysates were precleared and then incubated with GST-fusion proteins containing Mig-2 or filamin fragments for 2 hr or longer at 4°C. GST and GST fusion proteins were precipitated with glutathione-Sepharose beads and migfilin was detected by Western blotting with anti-migfilin mAbs.

Generation of Monoclonal Anti-Mig-2 and Migfilin Antibodies

Mouse mAbs recognizing Mig-2 and migfilin were prepared using GST fusion proteins containing Mig-2 residues 218–486 and migfilin residues 1–189, respectively, as antigens based on a previously described method (Tu et al., 2001, 1999). Hybridoma supernatants were initially screened for anti-Mig-2 and anti-migfilin activities by ELISA using MBP fusion proteins containing Mig-2 residues 218–486 and migfilin residues 1–189, respectively. Antibodies that recognize MBP-Mig-2 or MBP-migfilin in ELISA were selected and further tested by Western blotting using MBP-Mig-2, MBP-migfilin, and mammalian cell lysates.

Mammalian Mig-2 and Migfilin Expression Vector Construction and Transfection

DNA fragments encoding Mig-2 or migfilin sequences were cloned into the pEGFP-C2 vector (Clontech) or the pFLAG-CMV-2 vector (Sigma). Mouse C2C12 cells and human WI-38 cells were transfected with the GFP or FLAG expression vectors using LipofectAmine PLUS (Life Technologies) (Tu et al., 2001; Zhang et al., 2002). The expression of GFP- or FLAG-tagged proteins in the transfectants was confirmed by Western blotting.

Immunofluorescent Staining

Immunofluorescent staining was performed as described (Li et al., 1999; Zhang et al., 2002). Briefly, cells were plated on fibronectin-coated cover slips, fixed, and stained with either unconjugated or rhodamine- or FITC-conjugated mouse mAbs as specified in each experiment. Unconjugated mouse mAbs were detected with FITC- (in experiments in which cells were dually stained with rhodamine-phalloidin) or Rhodamine Red^{TX}-(in experiments in which cells were transfected with GFP-fusion proteins) conjugated anti-mouse IgG antibodies.

Coimmunoprecipitation

IMR-90 cells were lysed with 1% Triton X-100 in PBS [pH 7.1] containing 1 mM Na_3VO_4, 25 mM NaF and protease inhibitors. The cell lysates (450 μg) were incubated with the anti-migfilin mAb (5 μg IgG) or an irrelevant mouse IgG as a control for 2 hr. The samples were then mixed with 30 μl of UltraLink Immobilized Protein G (Pierce). After incubation for 2 hr, the beads were washed four times and the proteins bound were released from the beads by boiling in 50 μl of SDS-PAGE sample buffer for 5 min. The samples (25 μl/lane) were analyzed by Western blotting with anti-migfilin and anti-filamin mAbs, respectively. To immunoprecipitate GFP-fusion proteins, lysates (480 μg) of C2C12 cells transfected with vectors encoding GFP fusion proteins were mixed with 3.5 μl of rabbit anti-GFP antiserum (Clontech). The immunoprecipitates were analyzed by Western blotting with HRP-conjugated anti-GFP antibodies or mouse anti-filamin mAb and HRP-conjugated anti-mouse IgG antibodies.

RNA Interference

Sequences of Mig-2- and migfilin-silencing siRNAs were selected based on a previously described method (Elbashir et al., 2002). The targeted sequences that we found effectively mediate the silencing of the expression of Mig-2 and migfilin, respectively, are as follows (only sense sequences are shown): 5′-AACAGCGAGAAUCUUGG AGGC-3′ (Mig-2) and 5′-AAAGGGGCAUCCACAGACAUC-3′ (migfilin). The 21-nucleotide synthetic siRNA duplexes were prepared by Dharmacon Research. Hela cells were transfected with the Mig-2 specific siRNA, migfilin specific siRNA, or a 21-nucleotide irrelevant RNA as a control, using oligofectamine (Invitrogen) following the manufacturer's protocol. Suppression of Mig-2 and migfilin in the corresponding siRNA transfectants, but not the control RNA transfectants, was confirmed by Western blotting with anti-Mig-2 and migfilin mAbs.

Cell Spreading

Cells (as specified in each experiment) were plated in Opti-MEM I serum free medium (Life Technologies) in fibronectin (10 μg/ml) or laminin (10 μg/ml) coated 96-well plates. The plates were incubated at 37°C under a 5% CO2%–95% air atmosphere and the cell morphology was observed under an Olympus IX70 microscope and recorded with a digital camera. Unspread cells were defined as round cells, while spread cells were defined as cells with extended processes as described (Komoriya et al., 1991; Richardson et al., 1997; Tu et al., 2001; Zhang et al., 2002). The percentage of cells

adopting spread morphology was quantified by analyzing at least 300 cells from four randomly selected fields (>80 cells/field).

F Actin/G Actin In Vivo Assay

The ratio of F actin versus G actin in cells was analyzed using an F actin/G actin in vivo assay kit (Catalog Number BK037, Cytoskeleton Inc., Denver, CO) based on the manufacturer's protocol. Briefly, cells were lysed with a cell lysis and F actin stabilization (LAS) buffer and homogenized using 26.5 G syringes. The cell lysates were centrifuged at 100,000 g for 60 min at 37°C. The supernatants (G actin) were separated from the pellets (F actin) and were immediately placed on ice. The pellets were resuspended to the same volume as the supernatants using ice cold dH_2O containing 2 μM cytochalasin D and were incubated on ice for 60 min. Equal amount of the samples (2 μg of the G actin fractions and the corresponding amount of the F actin fractions) was loaded to each lane and analyzed by Western blotting with an anti-actin antibody. Actin in each fraction was analyzed using a chemiluminescence documentation system (Bio-Rad) and the ratio of F actin versus G actin was quantified using the Quantity One software (Bio-Rad).

Acknowledgments

We thank Yongjun Zhang, Tomohiko Fukuda, Hongwei Rao and Mary Ann Accavitti for technical assistance. This work was supported by NIH grants GM65188 and DK54639 to C.W.

Received: November 20, 2002
Revised: February 14, 2003
Accepted: February 17, 2003
Published: April 3, 2003

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Accession Numbers

The GenBank accession number for human migfilin reported in this paper is AY180161.